SUPPLEMENTAL MATERIAL

Extended Results

Note S1.

Consider a simple model of an epithelial cell, in which the apical membrane contains a Na^+ conductance (Ga^{Na}) and a Cl^- conductance (Ga^{Cl}) and the basolateral membrane contains a conductance (Gb). The cellular conductance for non-CF, Gc(non-CF), and CF, Gc(CF), is:

$$Gc(non-CF) = \frac{(Ga^{Na} + Ga^{Cl}) \cdot Gb}{(Ga^{Na} + Ga^{Cl}) + Gb}$$

$$Gc(CF) = \frac{Ga^{Na} \cdot Gb}{Ga^{Na} + Gb}$$

After amiloride treatment to block Ga^{Na} in non-CF epithelia, $Ga^{Na}=0$. Therefore:

$$Gc_{amiloride}(non-CF) = \frac{Ga^{Cl} \cdot Gb}{Ga^{Cl} + Gb}$$

$$Gc_{amiloride}(CF) = 0$$

Calculating the change in conductance produced by blocking the Na^+ conductance ($\Delta Gc_{amiloride}$) yields:

$$\Delta Gc_{amiloride}(non-CF) = \frac{(Ga^{Na} + Ga^{Cl}) \cdot Gb}{(Ga^{Na} + Ga^{Cl}) + Gb} - \frac{Ga^{Cl} \cdot Gb}{Ga^{Cl} + Gb}$$

$$\Delta Gc_{amiloride}(CF) = \frac{Ga^{Na} \cdot Gb}{Ga^{Na} + Gb} - 0$$

For constant values of Ga^{Na} and Gb, the $\Delta Gc_{amiloride}$ for non-CF cells will be less than for CF cells. That can be appreciated by considering the equation:

$$X = \Delta Gc_{amiloride}(CF) - \Delta Gc_{amiloride}(non-CF)$$

Solving this equation yields:

$$X = \frac{(G^{Na} \cdot G^{Cl} \cdot Gb) \cdot (G^{Na} + G^{Cl} + 2Gb)}{(G^{Na} + Gb)(G^{Cl} + Gb)(G^{Na} + G^{Cl} + Gb)}$$

X is a positive number, which indicates that:

$$\Delta Gc_{amiloride}(non-CF) < \Delta Gc_{amiloride}(CF)$$

Thus, in the absence of other changes, even if Na^+ conductance is the same in CF and non-CF cells, the $\Delta Gc_{amiloride}$, and hence $\Delta Gt_{amiloride}$ will be greater in CF.

Note S2.
In non-CF epithelia studied in the presence of amiloride and DIDS, forskolin and IBMX induced a greater Gt increase in nasal than tracheal/bronchial epithelia (Fig. 1F,1H), but a greater Isc increase in tracheal/bronchial than nasal epithelia (Fig. 1E,1G). Likewise, inhibiting CFTR with GlyH-101 produced greater Gt reductions in nasal than tracheal/bronchial epithelia (Fig. 1J,1L), but a greater Isc decrease in tracheal/bronchial than nasal epithelia (Fig. 1I,1K).

We considered several explanations for this difference between CFTR-mediated current and conductance. Because basolateral K^+ channels provide the electrical driving force for Cl^- secretion, tracheal epithelia might have more active K^+ channels than nasal epithelia. To test this possibility, we permeabilized the apical membrane and found a larger K^+ current in cultured tracheal than nasal epithelia (Fig. S3A). In addition, there were more transcripts for KvLQT1, a basolateral K^+ channel involved in airway epithelial Cl^- transport, in tracheal than nasal epithelia (Fig. S3B) (Kim et al., 2007; Mall et al., 2000). mRNA for KCa3.1 K^+ channels, which is also expressed in airway epithelia, did not differ at the two sites (Fig. S3C) (Bardou et al., 2009). These data suggest that compared to nasal epithelia, tracheal epithelia may have larger CFTR-mediated Cl^- currents because a greater basolateral K^+ permeability hyperpolarizes membrane voltage and creates a greater driving force for Cl^- secretion.

We also tested other hypotheses about the difference between the Isc and Gt data, but they showed no genotype-dependent difference. First, nasal epithelia might be more involved in transepithelial HCO_3^- than Cl^- transport, and performing the studies in HCO_3^--free solutions might have masked this possibility. However, when we used Cl^--free HCO_3^--containing solution, the cAMP-stimulated Isc was still greater in tracheal than nasal epithelia (Fig. S3D). Second, nasal epithelia might have a large basolateral Cl^- conductance that shunts Cl^- entering across the basolateral membrane back to the submucosal solution. If that were the case, inhibiting basolateral Cl^- channels should increase Isc. Therefore, we added DIDS, which inhibits most non-CFTR Cl^- channels, or GlyH-101 to the basolateral solution and found that neither inhibitor increased Isc in nasal epithelia; ΔIsc induced by basolateral DIDS was -3.4 ± 0.3 $\mu A.cm^{-2}$ (n = 3 nasal epithelia) and that induced by GlyH-101 was -0.6 ± 0.3 $\mu A.cm^{-2}$ (n = 3 nasal epithelia). Third, low levels of the Na^+-K^+-$2Cl^-$ cotransporter NKCC (Flagella et al., 1999) might limit transepithelial Cl^- transport in nasal epithelia. However, NKCC transcripts were actually 4-fold greater in nasal than tracheal epithelia (Fig. S3E).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES
Measurement of transepithelial voltage *in vivo*
Transepithelial voltage (Vt) was measured in the nose and trachea of newborn pigs using a standard protocol as described previously (Rogers et al., 2008; Standaert et al., 2004). Animals were anesthetized with propofol and breathed spontaneously during the measurements. Ringer's solution contained (in mM): 148 NaCl, 2.4 KH_2PO_4, 0.4 K_2PO_4, 2.25 $CaCl_2$, and 1.2 $MgCl_2$ (pH 7.4). Cl^--free Ringer's solution contained gluconate substituted for Cl^-. The reference electrode was a small Ringer's solution-filled catheter inserted into the leg muscle (25 g. needle). The exploring electrode was a 6 french foley catheter filled with Ringer's solution and placed onto the surface of the nasal cavity. We tilted down the head to allow liquid to drip from the nares and thereby minimize spread of solution onto the olfactory epithelium, which lies far from the site of measurement in the posterior part of the nose. Tilting down the head also prevented perfusate from entering the lung. To measure tracheal Vt, we made a small incision in the ventral portion of the trachea and placed the exploring electrode near the anterior trachea. We

perfused solutions in the following sequence: 1) Ringer's solution to obtain baseline Vt; 2) Ringer's solution containing 100 μM amiloride, 3) Cl⁻-free Ringer's solution containing amiloride plus 10 μM isoproterenol, 4) Cl⁻-free Ringer's solution containing amiloride, isoproterenol, and 100 μM ATP, 5) Cl⁻-free Ringer's solution containing amiloride, isoproterenol, ATP, and 100 μM GlyH-101. Each solution was perfused for 3-5 min at a rate of 5 ml/min. Vt was measured with a voltmeter connected to a strip chart recorder. Following completion of the measurements, the epithelium was disrupted by brushing and Vt measured again to determine the zero value of Vt.

Preparation of differentiated primary cultures of airway epithelia
Epithelial cells were isolated from the various tissues by enzymatic digestion and seeded onto collagen-coated, semi-permeable membranes ($0.6\ cm^2$ Millicel-PCF; Millipore, Bedford, MA) and grown at the air-liquid interface as previously described (Karp et al., 2002). Culture medium, a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12), was supplemented with 2% Ultroser G (BioSepra; Villeneuve, France). Differentiated epithelia were used at least 14 days after seeding.

Electrophysiological measurements in freshly excised and cultured epithelia
Epithelial tissues were excised from the nasal turbinate and septum, and from trachea through 2nd generation bronchi immediately after animals were euthanized. Tissues were mounted in modified Ussing chambers (area, $0.03\ cm^2$) (Physiologic Instruments, Inc., San Diego, Ca). Cultured epithelia were studied in a modified Ussing chamber (Jim's Instrument Manufacturing, Iowa City, IA) connected to a current-voltage clamp (Bioengineering, University of Iowa).

Epithelia were bathed on both surfaces with solution containing (mM): 135 NaCl, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 dextrose, 5 HEPES (pH = 7.4) at 37 °C and gassed with compressed air. For Cl⁻-free solution, Cl⁻ was replaced with gluconate and Ca^{2+} was increased to 5 mM. For the high K^+ and Na^+-free solution, Na^+ was replaced with K^+. To study HCO_3^- transport, we used Cl⁻-free Kreb's solution containing (mM): 118.9 NaGluconate, 25 $NaHCO_3$, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 5 CaGluconate, 1 MgGluconate, and 5 dextrose and gassed with 5% CO_2.

Vt was maintained at 0 mV to measure short-circuit current (Isc). Transepithelial electrical conductance (Gt) was measured by intermittently clamping Vt to +5 and/or -5 mV. Spontaneous values of Vt were measured by transiently removing the voltage clamp. A standard protocol used for epithelia from all sites was the following. 1) Measurements under basal conditions. 2) 100 μM apical amiloride to inhibit ENaC Na^+ channels. 3) 100 μM apical DIDS (4,4-diisothiocyano stilbene-2,2-disulfonic acid) to inhibit most anion channels other than CFTR. 4) 10 μM forskolin and 100 μM IBMX (3-isobutyl-2-methylxanthine) to increase cellular levels of cAMP leading to phosphorylation and activation of CFTR. 5) 100 μM apical GlyH-101 to inhibit CFTR. At the beginning of these experiments, we used cultured, non-CF tracheal epithelia to test the dose-response relationship for the agents used in this study (Fig. S1).

Measurement of Na^+ flux and fluid transport
Transepithelial Na^+ flux was measured using methods similar to those we previously reported (Flynn et al., 2009; Zabner et al., 1998). Epithelia were bathed with 500 μl fresh culture medium on the basolateral surface and on the apical surface with 250 μl solution containing (in mM): 137.8 NaCl, 4 KCl, 29 $NaHCO_3$, 1.2 $CaCl_2$, 0.6 $MgCl_2$, and 1 NaH_2PO_4. Osmolarity of the

solution was measured with a vapor pressure osmometer (WescorInc., Logan, UT) and adjusted with mannitol (~1 mM) to equal that of the culture medium. Prior to addition to epithelia, solutions were equilibrated in the culture incubator at 37 °C with 5% CO_2 and saturating humidity. To minimize evaporation, we filled the incubator with stacks of dishes that contain water and water-wetted spill pads. In addition, in each 24-well plate, 12 wells were used for experiments, and the other 12 wells were filled with 1 ml of saline solution. We assessed evaporation by collecting 1 μl of the liquid to which $^{22}Na^+$ was added; after 6 hr, the radioactivity was 101.7±1.4% of the starting value, suggesting very little if any evaporation.

For measuring Na^+ fluxes, we used pairs of epithelia from the same animal to study the two unidirectional fluxes of Na^+. For measuring $J^{Na^+}_{ap-bl}$, the apical solution contained 0.2 μCi ^{22}Na, and for measuring $J^{Na^+}_{bl-ap}$, the basolateral solution contained 0.4 μCi $^{22}Na^+$. After adding isotope, epithelia were incubated for 45 min to attain an equilibrium rate of transepithelial Na^+ flux. Then, apical (50 μl) or basolateral (100 μl) samples were collected at 0, 1, 2, and 3 hr. Amiloride (100 μM) was then added to the mucosal solution and samples were collected for an additional 3 hrs. After each sample, the same volume of non-radioactive solution was immediately added back to the epithelia. Radioactivity was measured using a liquid scintillation counter (LS6000SC; Beckman Coulter), and the data were used to calculate unidirectional and net Na^+ fluxes.

The methods for measuring liquid absorption were similar to those used for the Na^+ flux experiments, except that 120 μl of solution was placed on the apical surface. The volume of solution remaining on the apical surface was measured after 3 hr under basal conditions and then 3 hr after treatment with amiloride. Other details are as described previously (Zabner et al., 1998).

Quantitative real-time RT-PCR
Total RNA from excised tissue and cultures was isolated using RNeasy kit (Qiagen). RNA (0.5 μg) was reverse transcribed to synthesize cDNA (RT^2 first strand kit, C-03; SuperArray Biosciences). Table S1 shows the PCR primers; they were synthesized by Integrated DNA Technologies, Inc. All primer pairs were tested in PCR reactions to ensure that they only generated a single band of DNA segments. In addition, the amplified products were incorporated into plasmids (TOPO TA Cloning Kit, Invitrogen) and sequenced (DNA facility, University of Iowa) to confirm the correct identity.

Real-time RT-PCR was performed using RT² SYBR$^®$ Green qPCR Master Mixes (SuperArray Biosciences) and a 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA). Each sample was run in triplicate. All reaction products had dissociation curves with one peak and were further examined by agarose gel electrophoresis. Data were analyzed using Real-Time PCR Miner Program to derive the threshold cycle (Ct) and the efficiency of primer pairs. Data comparison was performed using the ΔΔCt method (Schmittgen and Livak, 2008). Expression of GAPDH served as an internal control. All data presented had Ct values < 35 cycles.

SUPPLEMENTAL FIGURE LEGENDS
Figure S1. **Relationships between Isc and concentration of inhibitors of electrolyte transport.** Data are mean ± SEM from cultured tracheal epithelia. **A**. Amiloride, an inhibitor of epithelial Na^+ channels (ENaC), was added to the apical surface under basal conditions. n= 5.

The concentration of amiloride that inhibited 50% of Isc (IC50) was 3.8±0.3 µM, which is close to the value obtained in Vt measurement *in vivo* (~2-3 µM in normal subjects and CF patients) (Knowles et al., 1983) and in excised human nasal tissue (~1 µM) (Mall et al., 1998). **B**. DIDS, an inhibitor of many non-CFTR Cl⁻ channels including Ca^{2+}-activated Cl⁻ channels (Schroeder et al., 2008; Yang et al., 2008), was added to the apical surface in the presence of 100 µM apical amiloride. n= 6. Adding apical DIDS inhibited only a small amount of current; note the difference in scale in panel B *vs.* the other panels. **C.** Earlier studies indicated that GlyH-101 (Muanprasat et al., 2004) inhibited porcine CFTR, whereas another CFTR inhibitor $CFTR_{inh}$-172 had smaller effects (Liu et al., 2007; Salinas et al., 2004). We added GlyH-101 to the apical surface of epithelia in the presence of apical 100 µM amiloride, apical 100 µM DIDS, 10 µM forskolin, and 100 µM IBMX. n = 5. The highest concentration of GlyH-101 (100 µM) inhibited 56±7% of the Isc. Higher concentrations of GlyH-101 and even 100 µM GlyH-101 caused a slow and gradual inhibition of transepithelial currents, including amiloride-sensitive currents, suggesting that it may also inhibit other epithelial ion channels as has been previously suggested (Caputo et al., 2008; Muanprasat et al., 2004). Consistent with that conclusion, 100 µM GlyH-101 slowly reduced current in CF epithelia, which do not have CFTR.

Figure S2. Amiloride alters electrical properties in non-CF and CF tracheal/bronchial epithelia. Data are means ± SEM from $CFTR^{+/+}$ (open symbols and bars) and $CFTR^{-/-}$ (closed symbols and bars) pigs. Numbers in parentheses indicate n, and * indicates P<0.05. **A-F**. Effects of adding amiloride (100 µM) to the apical solution on Vt, Isc and Gt of freshly excised (A-C) and differentiated primary cultures (D-F) of tracheal/bronchial epithelia. ΔVt_{amil}, ΔIsc_{amil}, and ΔGt_{amil} indicate changes induced by amiloride.

Figure S3. Non-CF tracheal/bronchial epithelia have larger Cl⁻ currents than nasal epithelia. Data are means ± SEM from nasal (cross-hatched bars) and tracheal/bronchial (shaded bars) epithelia. Amiloride (100 µM) was present on the apical surface in all cases. Numbers in parentheses indicate n, and asterisks indicate P<0.05. **A**. Basolateral K^+ currents in cultured non-CF epithelia. Solutions were Na^+-free and Cl⁻-free bilaterally, apical solution was K^+-free, basolateral solution contained 140.4 mM K^+, and apical solution contained forskolin and IBMX. Data are change in current following permeabilization of apical membrane with nystatin (0.36 mg.ml⁻¹). **B,C**. Relative KvLQT1 and KCa3.1 mRNA by q-RT-PCR in primary cultures of non-CF epithelia. **D**. $\Delta Isc_{F\&I}$ in epithelia bathed in Cl⁻-free HCO_3^- solution. **E**. Relative NKCC1 ($Na^+/K^+/2Cl^-$ cotransporter) mRNA by q-RT-PCR in primary cultures of non-CF epithelia.
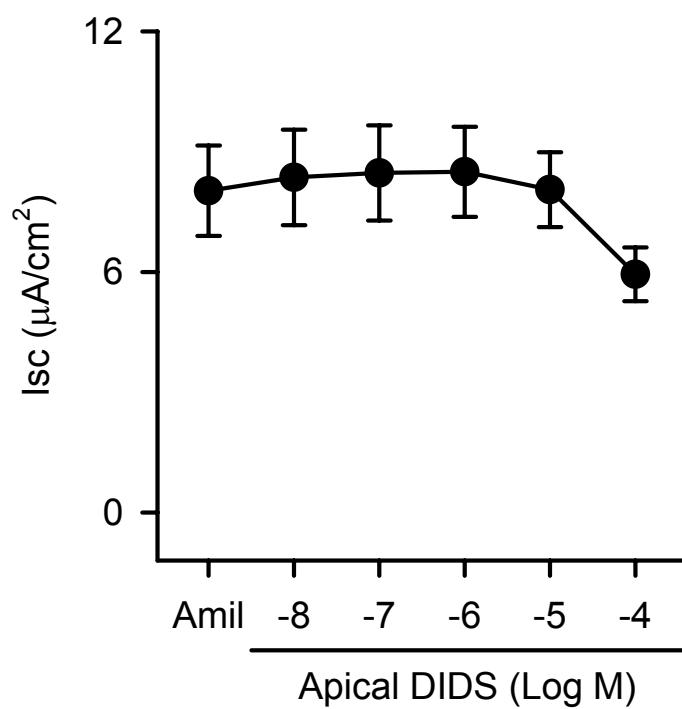
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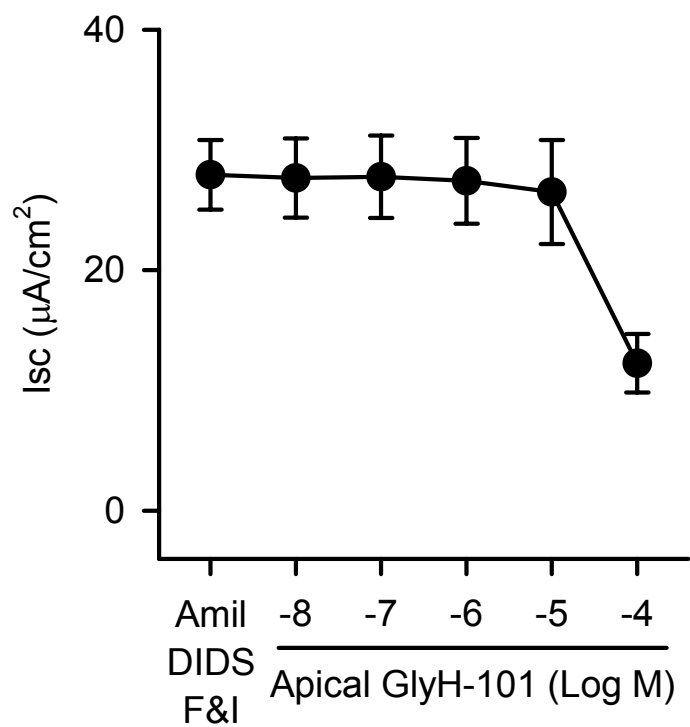
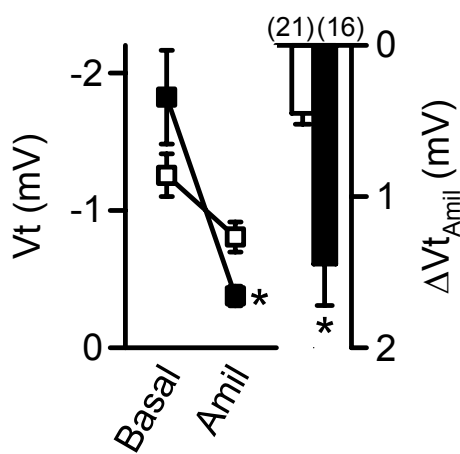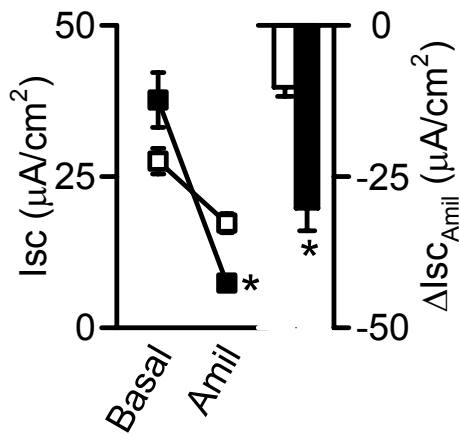Figure S2